

The University of Chicago

THE EFFECT OF LIVER EXTRACTS UPON ERYTHROPOIESIS IN THE CHICK EMBRYO

A PART OF A DISSERTATION SUBMITTED
TO THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

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By
EDWIN E. HAYS

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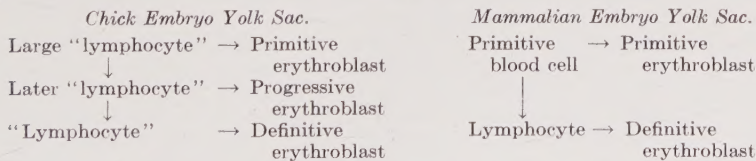
THE concept that the red cells in pernicious anemia are morphologically similar to those found in the embryo has been the basis of several attempts to^{4,6,12} devise an assay method for the antipernicious anemia factor.

The embryonic chick has the advantage that it is possible to direct the active liver fraction to the embryo itself without depending upon placental transfer, and the uncertainty of absolute age of the embryo, as is the case in mammalian studies, is practically eliminated. The erythropoietic organ, the yolk sac, is also readily available for study in contrast to that in mammals. The availability of the experimental animal cannot be overlooked.

Reimer⁹ studied the 9- to 15-day embryo. In his hands small doses had no effect upon the formation of erythroid elements in the bone marrow. Sabin,^{10a} using *in vivo* mounts of 3-day chick embryos, reported that active material prepared by Cohn caused a second division of primitive megablasts in the blood islands. Muller⁸ failed to confirm this finding.

These considerations led us to undertake the study of various potent liver fractions upon the chick embryo during the formation of the definitive, or mature, generation of red blood cells and their replacement of the primitive generation in the circulating blood and/or effect upon the blood at the site of its formation in the blood islands of the yolk sac.

According to Dantschakoff¹ the comparison between chick and mammalian erythropoiesis is indicated by this diagram:



* I wish to extend my gratitude to Armour & Co. for the grant under which these studies were made. I also wish to express my thanks to the Abbott Laboratories, Chappell Brothers, Lederle Laboratories, Inc., Ely Lilly and Company, and Wilson Laboratories, as well as Armour & Co., for the quantities of active liver preparations they kindly supplied for these studies.

The early forms of the primitive erythroblast are the cells regarded as morphologically similar to the megaloblast found in human pernicious anemia. Clear-cut differentiation of primitive erythroid types in the yolk sac is admittedly difficult and hence the possibility of changes in the yolk sac being reflected in the circulating blood was examined first.

From Sabin's^{10b} studies on *in vivo* mounts, it is known that granulocytes begin to wander into the circulating blood after the third day of incubation, the circulating lymphocytes (mature lymphocytic elements as contrasted with the erythroid elements of Dantschakoff) appear during the fourth day. These cells occur in small clumps in whole blood smears and should be readily distinguished from immature red cell forms.

If an acceleration in the rate of maturation or production of the definitive generation could be produced as a specific result of the injection of anti-anemia preparations, this change might be observed in the circulating blood at the proper transitional stage as well as in the yolk sac itself. This type of transformation is considered by some to be the mechanism involved in the response by anemia patients to liver extract.

If the circulating blood does not show any change, then the cytologic structure of the cells in the yolk sac might indicate whether there has been a stimulation in the production of the more mature cells without their release into the blood stream.

Methods. *Injection.* All injections were made under sterile conditions in a tissue-culture transfer chamber. The egg was placed with its pointed end upwards and after carefully wiping with 70% alcohol was pierced with a sterile lancet. The liver extract was injected directly into the egg white by means of a syringe through a short needle. After injection, the hole was sealed with boiled paraffin having a melting point between 50° and 55° C. The volume injected varied from 0.05 to 0.5 cc., however the majority of injections were 0.2 cc. All samples were rendered phenol-free by repeated vacuum concentration and dilution with water and were sterilized by means of a pressure Seitz filter.³ Fertile Redrock eggs were used and were incubated at 102° F. in a constant temperature incubator.

Preparation of Smears and Yolk Sacs. The eggs were candled and set so that the embryo floated on top and the shell carefully chipped away in order to expose the *area vasculosa* without breaking the yolk sac. A large vessel, preferably an artery, was carefully pricked with a fine needle and the blood drawn into a capillary pipette. Smears were made in triplicate using the slide and coverslip method and were stained with May-Grünwald-Giemsa stain. Five hundred consecutive cells were counted on each slide.

In later studies, after obtaining the blood sample, portions of the yolk sac were excised, spread on a small watch-glass, and immediately fixed with Zenker-formalin for 20 minutes. These were stained with hematoxylin-eosin-azure II according to the technique of Maximow, as described by Loosli.⁷ They were mounted in balsam.

Means of Evaluation. The possibilities for an evaluation of the preparations were: 1, the determination of the rate of maturation of the red cells of the different generations; and 2, the histologic differentiation of the cells in the blood-forming organs.

The method most practical for determining an increased maturation of red cell types in the circulating blood was that of differential cell counts upon smears. Since the definitives are easily distinguished from the primitives in such smears, an increase in their concentration at a given stage should indicate an increased formation with a corresponding increased maturation of the primitives which they replace. The source and cause of the difference could be determined in the yolk sac preparations.

In all cases the embryos were weighed in order to determine age.

Experimental. Preliminary studies showed that the definitives occur only occasionally in the circulating blood before $4\frac{1}{2}$ days of incubation and that their replacement of the primitives may be indicated by the percentage of definitives in the total number of cells. Hence, the 5 days' incubation was chosen as best suited for study.

Doses of 7 human units injected before incubation produced a pronounced increase in definitives over the untreated controls. Different commercial liver preparations of known therapeutic activity were injected at different levels. Extracts prepared from heart muscle and yeast were also used. Table 1 summarizes the results.

TABLE 1.—ERYTHROPOIESIS IN CHICK EMBRYOS INJECTED BEFORE INCUBATION.

Extract.	No. of units.	Total solids (mg.).	No. of embryos.	Average weight (mg.).	% embryos with over 50 definitives per 1000 cells.	% "re-sponse."*
None (controls)	..	..	160	182	11	
Saline	..	..	13	186	8	
Bacto-peptone	..	145.0	13	188	39	255
Difco Beef Extract	..	112.0	5	181	20	8
		66.2	6	184	0	
Yeast	..	118.0	5	190	0	
Whole stomach	..	63.0	15	176	36	227
Heart	..	64.5	8	183	12	
Lederle Liver Extract	2.0	15.9	20	194	10	
Lot No. 381H243S	3.0	26.0	7	178	35	218
	5.3	47.1	26	180	45	309
	7.1	58.0	57	177	51	364
	11.3	96.2	4	178	50	355
	13.5	113.0	12	175	50	355
	15.0	130.2	7	176	84	684
	18.0	181.5†				
Abbott	7.5	78.0	11	176	55	400

* Percentage over controls obtained by subtracting 11 from the value and dividing by 11.

† All died.

In an attempt to decrease the dosage required per chick embryo, injections were made directly on top of the area vasculosa at various stages of incubation. Injection at the third day of incubation appeared to give the best response. Table 2 summarizes the results.

It is apparent from Tables 1 and 2 that the response is non-specific and erratic. The question arises as to the significance of the values noted in Table 1. If the frequency of distribution is plotted for all 551 control smears, Table 3 results. According to

Fisher² only experimental values which lie outside of the range which includes at least 90% of all the control values are statistically significant. Applying this criterion to the above series of controls only smears having counts greater than 50 definitive generation cells per 1000 might be significant. This criterion eliminates most of the values listed in Tables 1 and 2. It is also obvious that the response is non-specific and not of quantitative value.

TABLE 2.—ERYTHROPOIESIS IN CHICK EMBRYOS INJECTED ON THIRD DAY OF INCUBATION ON AREA VASCULOSA.

Extract.	Total solids (mg.).	No. of embryos.	Average weight (mg.).	% over 50 definitives per 1000 cells.	% "response."
None (controls)		158	179	14	
Saline		22	164	21	50
Yeast	2.2	5	178	60	328
	6.0	6	185	50	257
Heart muscle	1.1	7	190	70	400
Lederle Liver Extract, Lot	1.0	4	172	100	614
No. 381H335B	2.4	7	160	42	200
	3.7	6	170	66	371
	5.0	6	184	0	
	9.5	19	177	75	435
Same in saline	0.1	10	182	20	43
	1.0	3	190	66	371

TABLE 3.—FREQUENCY OF DISTRIBUTION IN 551 CONTROL SMEARS.

No. of definitives per 1000 cells.	% of total number of cells.	No. of definitives per 1000 cells.	% of total number of cells.
0	5.7	41- 50	3.8
1-10	40.8	51- 60	3.4
11-20	19.3	61- 70	2.2
21-30	10.5	71-100	2.2
31-40	10.2	>100	2.1

However, to strengthen the evidence that the above responses were not due to the antipernicious anemia principle, more purified fractions were made from liver extracts of tested potency, following techniques of Laland and Klem⁵ and Sladek and Kyer.¹¹

These fractions, together with others recorded in Table 4, were injected before incubation. These negative results give additional evidence as to the non-specificity of the response observed.

Yolk Sac Studies. Yolk sac preparations also were made of from 3- to 5½-day embryos at 12-hour intervals of uninjected controls and eggs injected with 10 human units before incubation. There was no clear-cut difference between the injected and the control preparations although the cell counts upon the blood smears showed 80% to have over 50 definitives per 1000. There was no increase in the number of more mature cell types present nor an earlier appearance of definitive cells. In some preparations there appeared to be an increase in the percentage of the definitive types, but this finding was not consistent in other preparations and in those taken from embryos 12 hours later.

TABLE 4.—EFFECT OF LIVER FRACTIONS ON ERYTHROPOIESIS IN CHICK EMBRYOS INJECTED BEFORE INCUBATION.

Extract.	No. of units.	Total solids (mg.).	No. of embryos.	Average weight (mg.).	% over 50 definitives per 1000 cells.	% "re-sponse."
None (controls)			235	176	14	
Armour Lot 17	2.0*	125.0	8	173	0	
Armour Lot 29	7.0	127.0	4	170	50	257
Fraction 5	9.7†	38.8	5	181	0	
Fraction 6	12.2†	73.4	15	178	18	3
Fraction 1A6	10.2†	102.0	16	174	40	186
Lederle Liver						
Lot 381H304A	10.3	67.0	6	171	16	1
Lot 381H335B	6.5	56.4	18	177	28	100
	11.0	93.0	12	184	16	1
	14.2	122.6	16	179	24	7
Lot 381H243S	7.2	58.6	15	175	62	343
Fraction 1L7		48.4	8	173	12	
Wilson	7.7	93.0	0†			
Lilly	8.4	112.6	2†			
Armour Lot 96	7.7	101.5	0†			

* Highest non-toxic dose.

† Calculated from clinical assay.

‡ Toxic.

Camera lucida measurements of the mean nuclear diameter of the red cells in blood smears taken from these injected chick embryos showed no significant changes as compared with the controls.

It appears from these studies that the occurrence of the definitives in the circulating blood was largely a pouring-out effect and not a specific stimulation of red cell formation or maturation.

Summary. 1. Injection of eggs prior to incubation with liver extracts containing 7 or more human antipernicious anemia units produced an increase in the number of early adult cells in the circulating blood of the 5-day chick embryo.

2. Injection prior to incubation with saline and extracts prepared from heart muscle, yeast, whole stomach, and with commercial beef muscle and peptone preparations produced little or no increase in the number of these cells.

3. Injection at the third day of incubation with yeast and heart extracts, saline, as well as low doses of liver extracts, produced a non-specific and irregular increase in the number of these cells in the 5-day chick embryo.

4. Injection prior to incubation with more purified liver extracts produced no increase in the number of these cells in the circulating blood of the 5-day embryo.

5. Injection prior to incubation with active liver extracts failed to produce a significant change in the mean nuclear diameter of the cells circulating in the blood of the 5-day chick embryo.

6. Study of the yolk sac preparations of these embryos showed no consistent change in the rate of maturation or formation during erythropoiesis in the yolk sac indicating that the response noted in the injected embryos was due to a pouring out of these immature forms.

7. Statistical analysis of the data showed that the response obtained was not significant.

Conclusion. Erythropoiesis in the 3- to 7-day-old chick embryo cannot be utilized as a specific means of assay for the antipernicious anemia potency in liver extracts.

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